Label-retaining Cells in the Bulge Region are Directed to Cell Death after Plucking, Followed by Healing from the Surviving Hair Germ

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Hair plucking is the most frequently used method of anagen induction within hair follicles. In this study, we found that plucking leads to the entire renewal of the follicular stem cell region of the mouse pelage follicle. Comparative histochemical analysis revealed that S100A4 protein was specifically distributed in the outer layer of the epithelial sac, which has been identified as the stem cell region of the pelage follicle, whereas the slow cycling cells that retained 5-bromo-2′-deoxyuridine label for 8 wk were located in the epithelial sac and also in the hair germ. Combined terminal deoxynucleotide transferase deoxyuridine triphosphate fluorescein nick end labeling method and immunohistochemistry revealed that positive cells were detected in the outer layer of the epithelial sac possessing both bromo-2′-deoxyuridine and S100A4 labels 4.5 h after plucking. No terminal deoxynucleotide transferase deoxyuridine triphosphate fluorescein nick end labeling signal, however, was observed in the hair germ. Serial inspection of the plucked follicle revealed that almost all regions of the epithelial sac became terminal deoxynucleotide transferase deoxyuridine triphosphate fluorescein nick end labeling positive 12 h after plucking. Terminal deoxynucleotide transferase deoxyuridine triphosphate fluorescein nick end labeling-positive cells ultimately degenerated without forming apoptotic bodies. Subsequently, the surviving label-retaining cells in the hair germ migrated upward to re-epithelialize the damaged portion. These results indicate that follicular stem cells in the epithelial sac underwent cell death after plucking. It is likely that the hair germ is responsible for the reconstruction of the stem cell region of the hair follicle. ***Key words: epithelial sac/hair follicle/S100 protein/slow-cycling cell/stem cell. J Invest Dermatol 119:1310–1316, 2002***

The maintenance of self-renewing tissues, including the epidermis and hair follicle, largely depends on stem cells. The follicular stem cells can be identified primarily as label retaining cells (LRC), representing their long-lived, slow-cycling nature *in vivo*. It was initially demonstrated that LRC are exclusively found in the bulge region that marks the lower end of the epithelial permanent portion (Cotsarelis *et al*, 1990). More recent observations using three-dimensional reconstruction methods revealed that the bulge region possessing the LRC corresponded to the epithelial sac of the first generation that was usually attached to the ventral side of the growing follicles in murine pelage (Morris and Potten, 1999). As the bulge cells are conserved during hair growth cycles, the maintenance of the cutaneous tissues is thought to depend on the presence of long-lived bulge cells. It was recently demonstrated that the stem cells in the bulge region provided the progenitor cells for regeneration of both the hair follicle and also skin epidermis (Taylor *et al*, 2000).

Hair regrowth had once been considered to rely on the hair germ located above the dermal papilla of the resting follicle. The hair germ is morphologically similar to the bulge cells, but it is distinct in its biochemical properties (Ito and Kizawa, 2001). Label-retaining assays demonstrated that LRC are primarily found in the bulge region, but not in the hair germ (Lavker *et al*, 1993; Morris and Potten, 1999; Taylor *et al*, 2000). The hierarchical dominance of the bulge cells, therefore, is generally accepted, and the hair germ is considered to be a more specialized population of cells involved in hair follicle formation.

Hair plucking during the telogen phase is the foremost experimental means of inducing synchronous anagen phase, and is traditionally recognized as the most specific and effective physical anagen-inducing agent (Collins, 1918; Silver and Chase, 1970). Plucking initially induces depression of cell growth, although it is followed by hyperproliferation within the entire region of the follicular epithelial remnant (Silver *et al*, 1967; Potten, 1972). As these initial responses are a plucking-specific event and not observed during spontaneous anagen induction (Silver *et al*, 1967; Potten, 1972), hair cycle investigations have been principally performed during the later stages after plucking, in which follicle development proceeds normally, as in spontaneous hair growth (Chase *et al*, 1951). Consequently, there have been no studies of the cellular response immediately after plucking.

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Abbreviations: LRC, label-retaining cells; TUNEL, terminal deoxynucleotide transferase deoxyuridine triphosphate fluorescein nick end labeling method.

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Recently, we focused on the distinct onset of the plucking-induced anagen phase (Ito and Kizawa, 2001) and found that most of the epithelial remnant around the bulge region was directed to cell death after plucking. It has, however, not been determined whether slowly cycling stem cells in the bulge region are also directed to cell death. If this is so, how does the disrupted bulge region heal?

In this study, we have re-evaluated the plucking experiment in terms of the follicular stem cells. We report here that the bulge cells, previously thought to be thoroughly protected, were induced into cell death by the plucking of club hairs. Moreover, the remaining hair germ cells migrate to re-epithelialize the damaged region, including the attachment site of the arrector pili muscle.

MATERIALS AND METHODS

Animal treatments and labeling procedures Pregnant C3H/HeNCrj mice were purchased from Charles River (Yokohama, Japan). Conventional club hair plucking was performed with the wax dressing method (Paus *et al*, 1990). Briefly, 8 wk old mice were painted with a mixture of wax and rosin, and the embedded dorsal fur was gently removed. The wax dressing was freshly prepared to avoid incomplete hair plucking. At various times after plucking, dorsal skin tissues were excised.

Labeling the nuclei of slow-cycling cells with 5-bromo-2′-deoxyuridine (BrdU) was performed according to a previously established method (Taylor *et al*, 2000). Briefly, neonatal mice were subcutaneously injected with BrdU (50 μg per g body weight) twice daily for 3 d, from the third day after birth. After chasing for 8 wk, conventional club hair plucking was performed as mentioned above. At various times after plucking, dorsal skin tissues were excised.

We developed a labeling procedure for transit amplifying cells in the upper follicular epithelium of the telogen follicle. Eight week old mice were intraperitoneally injected with BrdU twice daily for 5 d. We have previously determined that this protocol exclusively labels almost the entire region of the interfollicular and the upper follicular epithelium above the level of the sebaceous gland. Conventional club hair plucking was then performed as mentioned above. At various times after plucking, dorsal skin tissues were excised.

For the detection of proliferating cells during the re-epithelialization stage after plucking, a continuous pulse-labeling experiment was performed. Mice were administrated with BrdU (20 μg per g body weight) at 2 h intervals from 12 h after plucking via intraperitoneal injection. Groups were terminated 16, 24, and 30 h after plucking and dorsal skin tissues were excised.

In each case, to ensure optical microscopic analyses, tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline for 16 h, embedded in paraffin wax, and then 6 μm sections were prepared.

Simultaneous detection of S100 expression and BrdU-labeled cells A dual-labeling method was employed to detect S100 proteins and BrdU immunoreactivity in the same section. Deparaffinized sections were permeated using 20 μg proteinase K per ml (Roche, Mannheim, Germany) at 37°C for 10 min. Following postfixation, either affinity purified anti-S100A4 IgG (5 μg per ml; Ito and Kizawa, 2001) or anti-S100A6 anti-serum (100-fold dilution; Swant, Bellinzona, Switzerland) was applied at room temperature for 1 h, followed by three rinses in phosphate-buffered saline for 5 min each. For the fluorescent signal detection of S100A4 immunoreactivity, the sections were incubated with 10 μg per ml of Alexa Fluor488-labeled goat anti-rabbit IgG (Molecular Probe, Eugene, OR) for 1 h. Fluorescent S100A4 immunoreactivity was photographed at this stage. For the detection of S100A6 immunoreactivity, bound antibody was detected using biotinylated goat anti-rabbit IgG and avidin-biotinylated alkaline phosphatase complex (Vector Laboratories, Burlingame, CA). The S100A6 immunoreactivity was then visualized using Vector blue. The tissue sections were then placed in distilled water and treated with 2 M HCl for 1 h at 37°C, to denature nuclear DNA partially. After three rinses with phosphate-buffered saline, each pretreated tissue section was incubated with monoclonal rat anti-BrdU antibody (Harlan Sera-Laboratory, Loughborough, U.K.), diluted 1 : 500, at room temperature for 1 h. Bound antibodies were sequentially detected with biotinylated goat anti-rat IgG and avidin-biotinylated peroxidase complex (Vector Laboratories). Finally, immunoreactivity was visualized with diaminobenzidine.

For single detection of BrdU immunoreactivity, the tissue sections were permeated and nuclear DNA was partially denatured before application of BrdU antibody.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) reaction, followed by the detection of BrdU-LRC and S100A4-expressing cells The TUNEL reaction was carried out on plucked skin sections utilizing an *in situ* cell death detection kit (Roche) according to the supplier's manual. Combined TUNEL and immunohistochemistry for BrdU or S100A4 protein was performed when comparative analyses of both signals were required. In this case, once the fluorescent signal from the TUNEL reaction was photographed, the section was processed for either BrdU immunohistochemistry, as described above, or S100A4 immunostaining. S100A4 immunoreactivity was visualized with diaminobenzidine as previously described (Ito and Kizawa, 2001).

Transmission electron microscopy Skin specimens were cut into 1 mm^3 pieces, fixed at 4°C in 0.5% glutaraldehyde/1.5% paraformaldehyde for 16 h and then postfixed in 1% osmium tetroxide for 1 h. Following dehydration in an ethanol series and impregnation with propylene oxide, the tissue was embedded in PolyBed 812 (Polysciences, Warring, PA). Tissue sections were cut at 1 μm with a Reichert Ultracut (Reichert-Jung, Vienna, Austria) and stained with 1% toluidine blue. Appropriate areas of the tissue were then sectioned at 80–90 nm. These ultrathin sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined and photographed using a JEM-100CXII (Jeol, Tokyo, Japan).

Image processing For the determination of the sectioned surface area of the cells, digital images of hematoxylin and eosin (H&E) preparations were obtained using a PDMC Ie camera (Polaroid, Cambridge, MA) mounted on an Axiplan-2 microscope (Carl Zeiss, Jena, Germany). Outlines of each cell were marked on the image to enclose the sectioned areas of the cell. Each area was then digitally measured by Micro-Analyzer (Polaroid).

RESULTS

S100A4- and BrdU-labeled epithelial sac The location of BrdU LRC was examined at the age of 8 wk, when all the follicles were in the second, prolonged telogen phase. These second telogen follicles are composed of two fused epithelial sacs. One is derived from the first-generation follicle and the other from the second-generation follicle that surrounds the secondary hair germ and the dermal papilla (Chase, 1954). Consistent with previous observations (Cotsarelis *et al*, 1990; Morris and Potten, 1994; Taylor *et al*, 2000), LRC were observed in the first generation of the epithelial sac, referred to as the bulge area, including the attachment site of the arrector pili muscle (**Fig 1*a***). Close inspection showed that the secondary hair germ and the epithelial sac of the second-generation follicle also retained some BrdU. Very little background was observed in the other portion of the epithelium. No staining was ever observed with the animals that were not previously injected with BrdU (data not shown).

We have previously described the distribution of S100A4 protein in the epithelial sac of the telogen follicle (Ito and Kizawa, 2001). For a precise comparison between LRC localization and S100A4 distribution, we employed a dual-labeling method to detect S100A4 and BrdU immunoreactivity in the same tissue sections. S100A4 immunoreactivity was found only in LRC residing in the outer layer of the epithelial sac (**Fig 1*b***). We identified two epithelial populations that possessed BrdU label, however, but not S100A4 signal. One was the secondary hair germ, where some cells possessed a similar intensity of BrdU label to the outer layer of the epithelial sac. The other was the inner layer of the first-generation epithelial sac.

Plucking-induced cell death in the epithelial sac To examine whether plucking damaged the epithelial sac, we subjected plucked follicle sections to the TUNEL reaction followed by immunostaining with antibodies against S100A4 or BrdU. As the plucking of clubs regularly pulls out the inner layers (Silver *et al*, 1967), the outer layer of the composite follicle

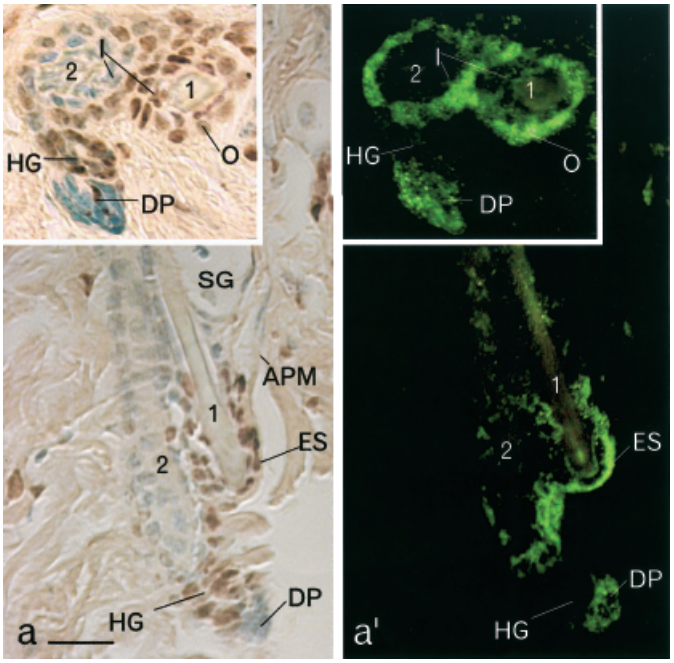

Figure 1. Co-localization of label retaining cells and S100A4 protein in the epithelial sac. A second-cycle telogen follicle at the age of 8 wk was subjected to dual labeling for S100A4 (*a*) and BrdU (*b*) immunoreactivity. *Insets* show a cross-section. BrdU immunoreactivity is confined to the outer layer of the second-generation sac (2), but is present in both layers of the first sac (1). S100A4 signal coincides with BrdU immunoreactivity in the outer layer of the sac. Note that BrdU label is present in the hair germ lacking S100A4 signal. S100A4 signal is also present in the condensed dermal papilla, adjacent to the hair germ. APM, arrector pili muscle; ES, epithelial sac; DP, dermal papilla; HG, hair germ; SG, sebaceous gland; I, inner; O, outer cellular layer of epithelial sac. *Scale bar*: 25 μm.

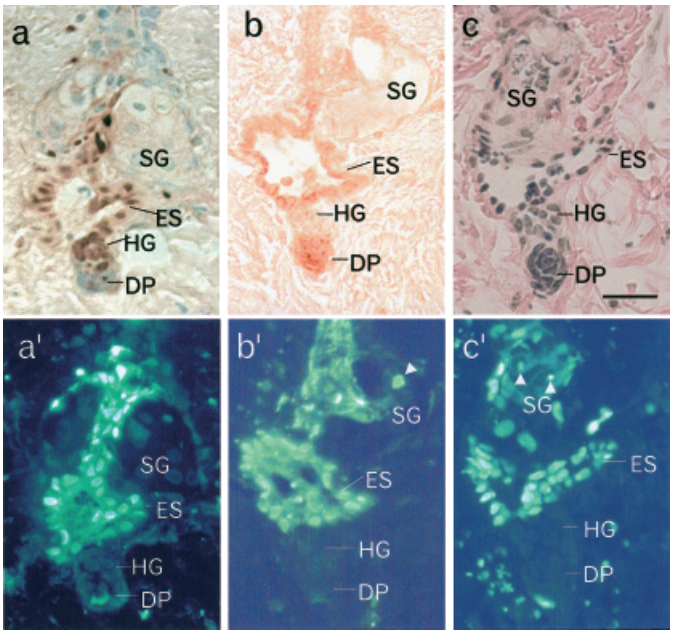

Figure 2. Identity of the epithelial sac and TUNEL-positive region. Dual-labeling of TUNEL reaction with BrdU LRC (*a,a′*), or S100A4 immunoreactivity (*b,b′*) was performed with skin tissue 4.5 h after plucking. Note that the clusters of BrdU label retaining cells and S100A4 expressing cells below the sebaceous gland (*a,b*) are ubiquitously positive for TUNEL staining (*a′,b′*). Hair germ and dermal papilla are constantly TUNEL negative. Twelve hours after plucking, mouse dorsal tissues were subjected to H&E staining (*c*), following the TUNEL reaction (*c′*). Note that the nuclei of the epithelial sac are densely stained by hematoxylin. *Arrowheads* show TUNEL-positive sebocytes. ES, epithelial sac; DP, dermal papilla; HG, hair germ; SG, sebaceous gland. *Scale bar*: 25 μm.

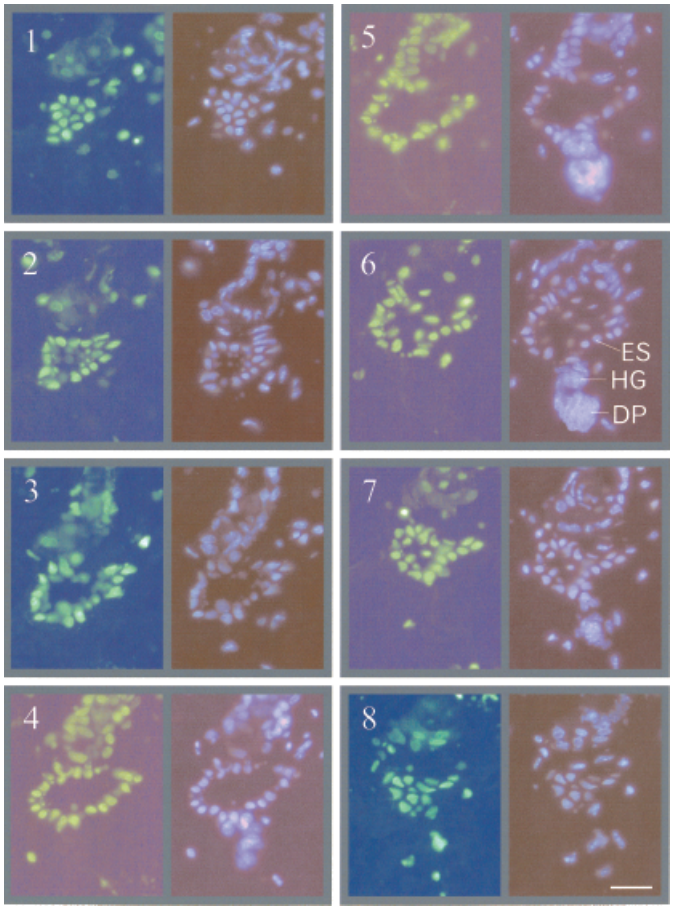

Figure 3. TUNEL reaction on the serial sections of a plucked follicle. TUNEL reaction was performed on the serial sections of a follicle 12 h after plucking club hairs (*left panels*). Serial numbers of the sections are indicated on the upper left of the micrograph. Nuclei were counterstained with Hoechst-33258 (*right panels*). Note that the nuclei of the entire region of the epithelial sac are TUNEL positive. No TUNEL-positive cells were seen in the hair germ and the dermal papilla. ES, epithelial sac; DP, dermal papilla; HG, hair germ. *Scale bar*: 25 μm.

was recognized as a single layered epithelium, continuous with the hair germ. At 4.5 h after plucking, the single layered epithelium was stained to detect BrdU (**Fig 2*a***) and S100A4 protein (**Fig 2*b***), confirming that this single layer corresponds to the epithelial sac. TUNEL-positive cells were detected in the epithelial sac, immunoreactive for both S100A4 and BrdU (**Fig 2*a′*,*b′***). In contrast, the secondary hair germ possessing some LRC, but not S100A4 protein, was negative for TUNEL. Importantly, analysis of H&E preparations revealed that the TUNEL-positive cells were not divided into morphologically distinct apoptotic bodies even 12 h after plucking (**Fig 2*c***). On the contrary, they looked almost normal with the exception of the nuclei, which were more densely stained with hematoxylin. At this time, serial inspection showed that virtually all regions of the epithelial sac were TUNEL positive (**Fig 3**, **Table I**). We could not detect S100A4 or BrdU in the TUNEL-positive region at this stage, however, which indicated that those molecules had been destroyed during cell death progression (data not shown). Intriguingly, serial inspection of H&E preparations of the later stage of plucked follicles revealed that the nuclei of almost all regions of the epithelial sac were not stained with hematoxylin (**Fig 4*a***). Transmission electron microscopy observation confirmed that the nuclei of the single epithelial layer, just above the hair germ, showed apparent degenerative changes (**Fig 4*b***).

LRC in the hair germ reconstruct the bulge region after plucking To investigate the healing process of the damaged

Table I. Induction of cell death in the stem cell region of the telogen follicle[a]

Hours after plucking	Percentage of epithelial sac possessing TUNEL-positive cells	Percentage of TUNEL-positive cells
0	0±0	0±0
4.5	100±0	70.3±5.5
12	100±0	99.7±0.5

[a]Twenty sections of the epithelial sac from two C3H mice were analyzed. Values represent the average percentage of 20 follicles±SD.

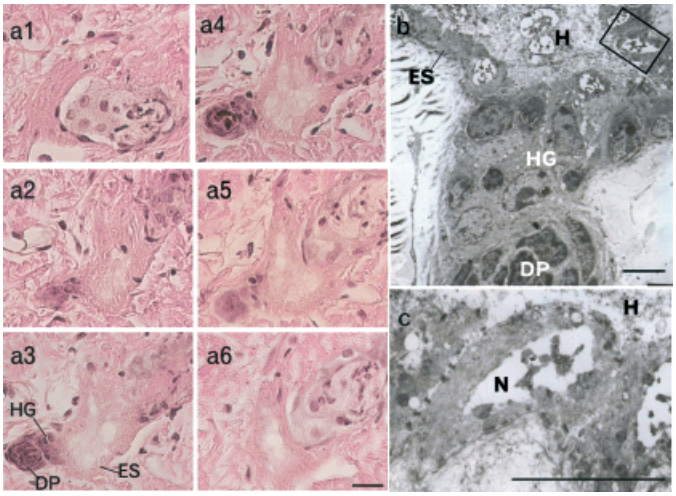

Figure 4. Morphologic analysis of the dead epithelial sac in plucked follicles. Serial sections of a follicle seen 18 h after plucking were stained with H&E. Serial numbers of the sections are indicated on the upper left of the micrograph. Note that the cells constituting outer layer of epithelial sac are solely stained with eosin, but not with hematoxylin (*a*). Electron micrograph of the plucked follicle at the same stage (*b*). Note that the degenerated cells possessing evenly disrupted heterochromatin are clustered in the epithelial sac. Boxed degenerated cells are shown in a higher magnification (*c*). The boundaries between nuclear membrane and cytoplasm are ill defined. Cell organelles, including Golgi apparatus and endoplasmic reticulum are not observed and some mitochondria are disrupted. DP, dermal papilla; ES, epithelial sac; H, plucking-induced hole in the follicle; HG, hair germ; N, nucleus. *Scale bar*: (*a*) 25 μm; (*b*,*c*) 5 μm.

follicle, we followed the fate of LRC in later stages postplucking. As the initial healing response was fairly uniform, a variety of re-epithelialization stages were observed in each follicle tissue during 18–30 h after plucking. In the early stages, the dead epithelial sac occupied a large part of the lower follicle, and BrdU immunoreactivity was diminished. Consequently, LRC were exclusively found in the hair germ region below the epithelial sac (**Fig 5*a***). In more advanced stages, the damaged region was partially repopulated with living cells. LRC were distributed in the regenerating lower follicle (**Fig 5*b***). The damaged portion was largely re-epithelialized by 30 h. BrdU-positive cells were still observed in the regenerated lower follicle (**Fig 5*c***). The reproducibility of cell death induction by plucking was high enough to confirm that LRC in the re-epithelialized region were not derived from the epithelial sac. These results demonstrated that the surviving LRC residing in the hair germ participated in the re-epithelialization of the damaged stem cell region.

Upper follicular epithelium are not concerned with re-epithelialization of the lower follicle It has been reported that the upper follicle, above the level of the sebaceous gland duct, consists of cells that are often replaced by mitosis even in the telogen phase (Lavker *et al*, 1993). On the other hand, the

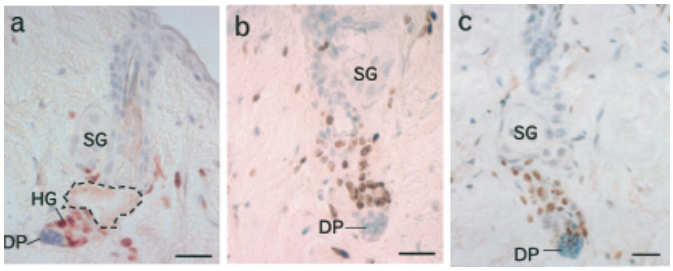

Figure 5. LRC in the hair germ migrated into the damaged portion after plucking. BrdU-LRC-labeled mice was subject to BrdU immunohistochemistry after plucking. Eighteen hours later, LRC are present in the expanding hair germ, below the dead epithelial sac enclosed by dashed line (*a*). LRC are seen in the partially re-epithelialized lower follicle 24 h later (*b*). Some LRC remain in the repaired bulge region 30 h later (*c*). DP, dermal papilla; HG, hair germ; SG, sebaceous gland. *Scale bar*: 25 μm.

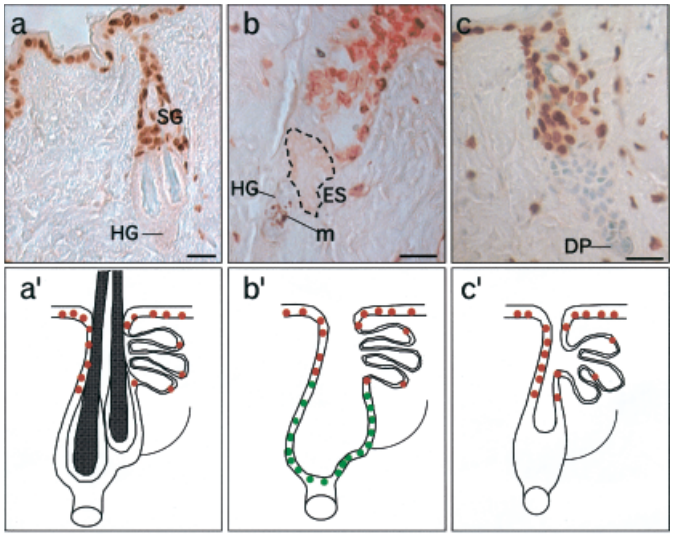

Figure 6. Damaged lower follicles were repaired without incorporation of the upper follicular cells. Most of the upper follicular cells were marked after administration of BrdU for 5 d during the telogen phase (*a*). Eighteen hours after plucking, the hair germ containing some melanin granules was observed below the de-nucleated dead epithelial sac enclosed by dashed line (*b*). Thirty hours after plucking, the destroyed bulge region was filled with living cells without BrdU label (*c*). The BrdU label localization at each stage is schematically illustrated below the micrographs. Note that BrdU-labeled cells remained above the sebaceous gland. DP, dermal papilla; HG, hair germ; M, melanin granules; SG, sebaceous gland. *Scale bar*: 25 μm.

lower portion, including both epithelial sac and hair germ, consists of mitotically inert cells (Bullough and Laurence, 1958). Taking advantage of this different kinetic property, we attempted to mark exclusively the upper follicular cells. Administration of BrdU to mice for 5 d during the telogen phase succeeded in selectively BrdU labeling almost the entire region of the upper follicular epithelium, without incorporation into the lower follicle (as shown in **Fig 6*a***). To examine whether the upper follicular epithelium migrates to heal the lower portion, we followed the fate of the BrdU label after plucking club hairs. Eighteen hours later, dead epithelial sac remnants were still observed above the hair germ without BrdU label (**Fig 6*b***). More than 70% of the follicles re-epithelialized the damaged portion within 30 h. BrdU-labeled cells, however, were not observed in the reconstructed bulge region, but remained in the upper part of the follicle (**Fig 6*c***). These results indicated that no transit amplifying cells residing in the upper portion of the telogen follicle migrated into the destroyed lower region, even though they are located adjacent to the dead cells.

Re-epithelialization of the lower follicle mainly relies on cell migration To investigate the proliferative activity during the healing of damaged follicles, we performed a continuous BrdU pulse-labeling experiment. Simultaneously, we analyzed expression of S100A6 protein, which is localized in the bulge region of the anagen follicle (Ito and Kizawa, 2001). Sixteen hours after plucking, the hair germ expressed S100A6 mRNA (data not shown), in agreement with a previous report (Ito and Kizawa, 2001). S100A6 protein, however, could not be detected in the hair germ aggregate, beneath the dead epithelial sac (**Fig 7*a***). S100A6 protein immunoreactivity was initially detected in the migrating tongue of the hair germ towards the damaged region (**Fig 7*b***). The S100A6-expressing region was gradually expanded as the re-epithelialization advanced, but proliferating pulse-labeled cells were not appreciably detected in the lower follicle by 24 h after plucking (**Fig 7*c***). Image analysis using H&E preparations of this stage revealed that the sectioned area of the re-epithelizing cells (**Fig 7*e***) was increased up to four times, compared with the hair germ cells, immediately after plucking (**Fig 7*f*, Table II**). Thirty hours after plucking, S100A6 protein was distributed all over the regenerated lower follicle (**Fig 7*d***). By this time, the pulse-labeled nuclei had accumulated in the lower follicle. The time course of the accumulation of BrdU incorporation in the lower regenerating follicle is summarized in **Table III**. The onset of the anagen phase, induced by plucking, has been defined as the first appearance of mitotic activity in the hair germ (Chase *et al*, 1951). This later stage of re-epithelialization also fulfills this definition, and therefore cannot be distinguished from the anagen phase as previously suggested (Silver *et al*, 1967).

DISCUSSION

Plucking directs LRC to cell death We have demonstrated that the follicular slow-cycling cells are directed to cell death upon hair plucking. Serial inspection of plucked follicle sections showed that almost all nuclei of cells located in the epithelial sac were stained using the TUNEL reaction. Subsequently, heterochromatin of these TUNEL-positive cells disappeared without forming apoptotic bodies. Thus, LRC certainly underwent cell death upon plucking. Given the significant attention to the stem cell region of the hair follicle, it is quite surprising that this striking phenomenon has not been reported previously.

Morris and Potten (1999) previously reported that highly persistent LRC, which retained label for 14 mo, remained in the bulge region after plucking. We could not find such a defined epithelial population, however, that escaped the massive cell death in the epithelial sac. The reason of this difference is unknown. As Morris and Potten, however, used the plucking procedure for anagen induction, without expecting cell death in the epithelial sac, it might be possible that the plucking cut off the hair before damaging the stem cell region.

As it has been thought that the bulge region must be highly protected in order to maintain the hair regenerative capacity, one might be surprised that hair plucking, which is the foremost inductive means of follicle regeneration, destroyed the bulge region. Cotsarelis *et al* (1990) proposed the bulge activation hypothesis, based on the observation that the bulge region is highly protected through physical hair cycling, as well as after hair plucking (Lavker and Sun, 1995). This appears inconsistent with our results, which show that the follicular stem cell region was vulnerable to plucking. It is noteworthy, however, that our results were the consequence of plucking club hairs that had already terminated their growth in the telogen follicle. The plucking of club hairs attaching to the anagen follicle similarly induced cell death in the epithelial sac (data not shown). On the other hand, plucking exclusively the growing hair out of the anagen follicle did not damage the adjacent epithelial sac, and the follicular stem cells in the sac remained intact (data not shown). Therefore, it is quite possible that plucking the growing hairs, but not the club hairs, might lead to the conclusion that the bulge region is not vulnerable to hair plucking. Furthermore, the degeneration of the epithelial sac might fail to be recognized, as the nuclei of the sac seem almost normal for some time after plucking and re-epithelialization of the damaged portion is

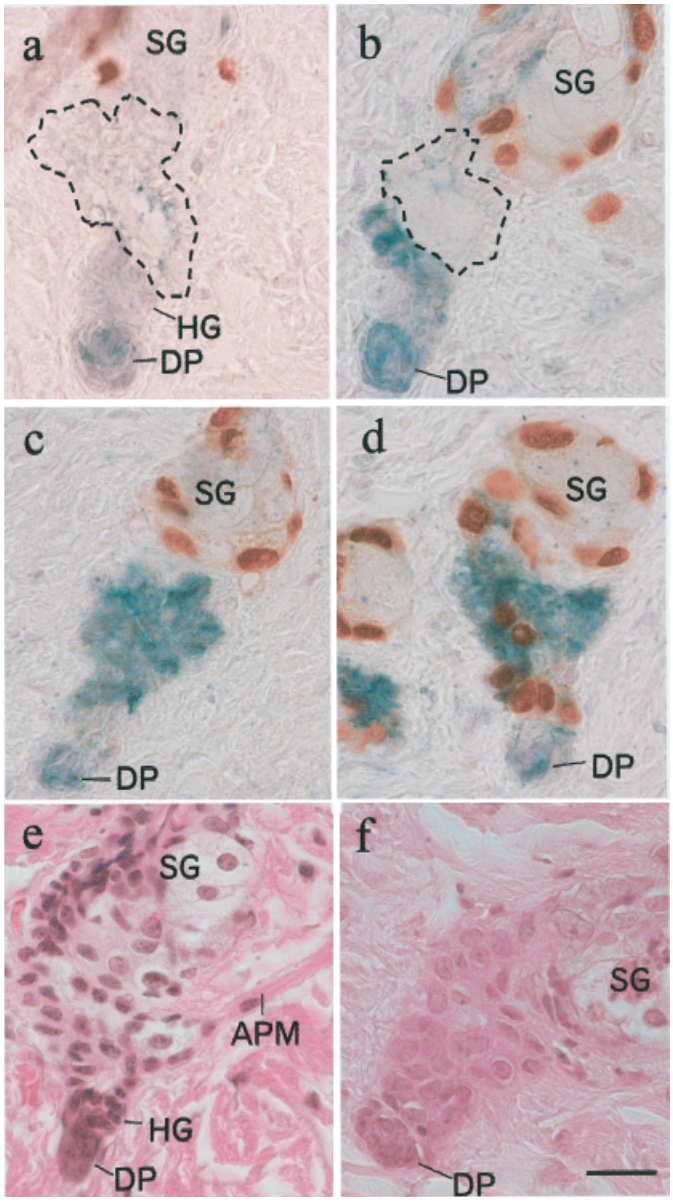

Figure 7. Distribution of pulse-labeled cells during repair of the lower follicle. Mice were administered BrdU continuously at 2 h intervals from 12 to 30 h after plucking. Tissues were subjected to combined immunohistochemistry for S100A6 protein and BrdU. Sixteen hours after plucking, S100A6 staining as well as BrdU label are rarely observed in the hair germ before upward migration (*a*). Twenty-four hours after plucking, the migrating tongue of the hair germ induced S100A6 expression, but BrdU-labeled cells were yet to be observed (*b*). In some follicles, 24 h after plucking, S100A6 protein localization is extended to the entire re-epithelialized region, but BrdU-labeled cells are not observed in this section (*c*). Thirty hours after plucking, BrdU-labeled cells are seen in the S100A6-expressing re-epithelialized region as well as the hair germ below (*d*). Note that S100A6 protein is also expressed in the dermal papilla. An H&E preparation of the follicle immediately after plucking (*e*). Note the small, tightly compacted cells in the hair germ. An H&E preparation of the follicle 24 h after plucking (*f*). Note the apparently enlarged cells in the lower follicle. DP, dermal papilla; HG, hair germ; SG, sebaceous gland.

Table II. Enlargement of the hair germ cells during upward migration

Hours after plucking	Sectioned area of each lower follicular cell (μm^2)[a]
0	22.1 ± 8.6[b]
24	85.0 ± 7.3[c]

[a]Values represent the average of 12 hair follicles from two different mice ± SD.
[b]More than 100 cells of the hair germ
[c]or the re-epithelialized lower follicle were measured.

Table III. Accumulation of pulse labeled cells in the re-epithelialized lower follicle[a]

Labeling period (hours after plucking)	Percentage of re-epithelialized lower follicle[b]	Total number of BrdU positive cells[c]
12–16	0 ± 0	0 ± 0
12–24	28.3 ± 5.8	1.3 ± 2.3
12–30	76.1 ± 5.8	48.7 ± 7.0

[a]Mice were continuously administrated with BrdU (20 μg per g body weight) at 2 h intervals from 12 h after plucking via intrapertoneal injection. After labeling for 4, 12, and 18 h, dorsal skin was excised from three mice in each group. Twenty lower follicle sections were analyzed for each mouse. Values represent the average of three mice ± SD.
[b]Lower follicle sections containing no damaged cells were scored as the re-epithelialized follicle.
[c]Counts represent total number of pulse-labeled cells during each labeling term in 20 follicle sections.

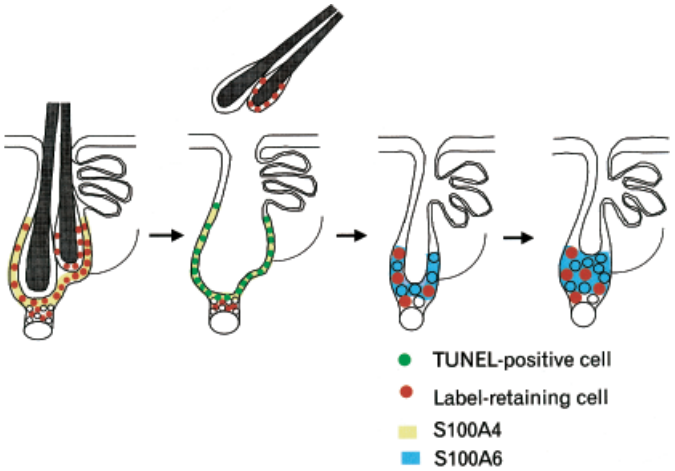

Figure 8. Schematic representation of the bulge reconstruction after plucking. LRC located in the epithelial sac possess S100A4 immunoreactivity. The second population of LRC is present in the hair germ without S100A4 immunoreactivity. The outer layer of the epithelial sac and the hair germ remain in the skin after plucking club hairs. As a consequence of the epithelial sac degeneration, LRC remain exclusively in the surviving hair germ. In the initial stage of repair process, hair germ cells with S100A6 protein expand and re-epithelialize the damaged follicle. Thereafter, the damaged region is filled with hair germ cells and their daughter cells.

accomplished within a few days. Thus, the re-epithelialized region may be considered as an intact bulge after plucking.

It was previously reported that follicular, slowly cycling, cells reside in the outer layer of a two-layered epithelial sac (Morris and Potten, 1999). It was also reported that plucking the club hair also pulls up the inner layer of the epithelial sac (Silver *et al*, 1967). Thus, it seems reasonable that the remaining outer layer harboring LRC is severely damaged by peeling off its adjacent inner layer. It is known that the interfollicular epithelium is directed to cell death when the upper layer is peeled off (Lessard *et al*, 1968; Potten and Allen, 1975). Although the precise mechanism of plucking-induced cell death is elusive at present, there might be some similarity with such epithelial wounds generally induced by cellular stripping.

LRC in the hair germ regenerate the damaged stem cell region Despite similar BrdU labeling and chasing term (8 wk), we detected LRC in almost all regions of the epithelial sac and the hair germ, whereas only a small number of LRC were identified exclusively in the bulge region by Taylor *et al* (2000). The reason for this difference might be due to the efficient antigen retrieval, and more sensitive detection procedure using the avidin and biotinylated enzyme complex system of our BrdU immunohistochemistry method. Although the number of stem cells in the hair follicle is uncertain, it might be possible that we also identified early progenitors of the stem cells, in addition to the stem cells. Nevertheless, the distribution of LRC in this study indicates that the epithelial sac and the hair germ are relatively slowly cycling compared with the other epithelial portion, where no BrdU signal was detected.

Strikingly, we revealed that LRC located in the hair germ were the only surviving label-retaining population in the plucked follicle. Some LRC were detected after healing of the lower follicle. In addition, no upper follicular epithelium migrated into the lower region. These results strongly suggest that the LRC in the hair germ were responsible for re-epithelialization of the damaged portion of the plucked follicle.

We showed that re-epithelialization of the plucked follicle mainly relies on an upward migration of the hair germ cells, coupled with cell size expansion, even though the later stage of the healing process involved significant mitotic activity. These results are consistent with previous observations showing that the plucking-induced cavity in the skin is filled with cells prior to extensive mitosis (Silver *et al*, 1967). It has been widely accepted that re-epithelialization of the injured epidermis is usually accomplished by a migration of remaining epithelium to cover the entire wounded area, prior to extensive cell mitosis (Potten and Allen, 1975). Thus, the healing process of the plucked follicle is similar to that of the wounded epidermis, as previously suggested (Silver *et al*, 1967).

Surrogate stem cells reside in the hair germ It has been demonstrated that a prolonged label chase period completely exclude LRC out of the hair germ, and long-term LRC were exclusively found in the epithelial sac (Morris and Potten, 1999). Moreover, others have demonstrated that the bulge region provides the progenitor cells that differentiate into the all epithelial portion of the hair follicle (Taylor *et al*, 2000; Oshima *et al*, 2001). Therefore, until this report, it has been considered that the follicular stem cells are primarily located in the epithelial sac corresponding to the bulge region and no stable population of stem cells resides in the hair germ (Wilson *et al*, 1994).

Surprisingly, we have demonstrated that the hair germ alone could solely regenerate the hair follicle, including the bulge region, after plucking. Absence of S100A4 expression from the LRC residing in the hair germ certainly confirmed that they are a distinct population from the bulge cells. Nevertheless, S100A6 protein, previously reported as expressed in the bulge region of the anagen follicle (Ito and Kizawa, 2001), was induced in the hair germ expanding toward the healing bulge region after plucking. Hence, we believe that the hair germ cells might serve as surrogate stem cells, which can easily migrate into the bulge after its destruction. The bulge reconstruction process, after plucking, is schematically represented in **Fig 8**. It has been reported that the progenitors of epithelial stem cells are capable of de-differentiation into the stem cells, if damaged, in the intestinal system (Booth and Potten, 2000). Although the actual hierarchical relationship of the hair germ and the bulge is elusive at present (Panteleyev *et al*, 2001), it seems likely that both epithelial populations have a potential plasticity to replenish each other, as in the intestinal system.

As hair plucking is a situation often encountered in daily life, the bulge reconstruction might be an important mechanism for the maintenance of the regenerative capacity of hair follicle. Further investigations utilizing plucking will hopefully lead to the better understanding of the stem cell constitution, as well as the control of the stem cell number in the hair follicle.

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